EXHIBIT 1

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Original Report

Individual Dosing of ASA Prophylaxis by Controlling Platelet Aggregation

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Summary: Acetylsalicylic acid is widely used in the primary and secondary prevention of cardiovascular diseases. In the current study, we used platelet aggregation ex vivo in platelet-rich plasma induced with arachidonic acid as a routine method for the determination of the individual dose of acetylsalicylic acid necessary to inhibit platelet aggregation in 108 patients with cardiovascular diseases. In 40% of all patients studied, a dose of 30 mg/day was sufficient to block the arachidonic acid–induced platelet aggregation nearly completely. In 50% of all patients, a dose of 100 mg/day was necessary. In 10% of all patients, the dose had to be further increased to 300 mg/day or even to 500 mg/day to inhibit platelet aggregation nearly completely. These results demonstrate that platelet aggregation can be used as a simple routine laboratory method to control acetylsalicylic acid treatment in patients with cardiovascular diseases and to determine individual doses of acetylsalicylic acid for a nearly complete inhibition of platelet aggregation. With a standard dose of 100 mg/day, 10% of the patients were nonresponders. **Key Words:** Platelet aggregation—Acetylsalicylic acid—Slope—Primary and secondary prevention—Cardiovascular diseases—Diabetes mellitus.

Acetylsalicylic acid (ASA) is widely used as an antiplatelet drug in the primary and secondary prevention of myocardial infarction, stroke, transient ischemic attacks, peripheral arterial disease, and other vascular diseases (1–4). It selectively inhibits the platelet prostaglandin G/H synthase with a loss of the cyclooxygenase activity and a suppression of the platelet thromboxane A2 biosynthesis, and in other tissues the formation of different eicosanoids (prostaglandin E2, prostacyclin) is inhibited (5–8). The antithrombotic effects of ASA are associated with the inhibition of platelet aggregation (PA) and the prevention of the deposition of platelets at atherosclerotic lesions (9–11). The optimal individual dose of ASA is still a matter of discussion (12–14). In 1999, a meta-regression analysis of the dose-response effect of ASA on stroke was reported. The conclusion was as follows: "The risk reduction of 15% is uniform across a wide range of doses (50 to 1,500 mg/day). The absence of a dose-response relationship supports the use of low-dose aspirin, because low-dose aspirin may minimize the risk of milder gastrointestinal tract toxic effects. The lowest effective aspirin dose has not yet been identified, but could be lower than 50 mg/day" (14, p. 1253). The most widely used method for the detection of platelet function is PA, as described in 1962 (15). The specific inhibition of PA induced by different agonists characterizes hereditary platelet dysfunctions, acquired abnormalities, and shows insight into the physiology and pathophysiology of platelets (16–18). The inhibition of PA induced by arachidonic acid (AA) shows the inhibition of the cyclooxygenase pathway in platelets (19,20). Therefore, we focused on the AA-induced PA in platelet-rich plasma (PRP), and investigated 108 patients with a medical indication for ASA in order to show that the ex vivo PA can be used as a simple routine method to control ASA treatment. In every patient, it is possible to define the minimal effective dose of ASA that leads to a nearly complete inhibition of PA.

PATIENTS AND METHODS

Subjects

One hundred eight patients of the district hospital of Stadtroda (Germany) with a medical indication for ASA were tested (52 men and 56 women; aged 46 to 93 years; 73.5 ± 1 standard error of the mean [SEM]). None had

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taken ASA or other nonsteroidal antiinflammatory drugs including Cox-2-inhibitors for at least 2 weeks before the first blood sampling. All patients had a normal platelet count between 150,000 and 350,000 μL (227,000 ± 6,000 μL SEM) and had no bleeding problems or gastrointestinal tract abnormalities in their history.

Blood collection and platelet preparation

Blood was drawn from patients with an empty stomach from the antecubital vein and, in some cases, from other veins of the arms during the routine blood collection for other laboratory determinations between 7:00 and 8:00 AM. The first 5 mL of blood was discarded or used for other chemical determinations. The tourniquet had been loosened or reduced to minimize platelet activation. For platelet aggregation, 4.5 mL of blood was drawn into commercially available tubes (nine parts whole blood, one part sodium citrate (3.8%) as an anticoagulant; final concentration 0.11 mol/L, Becton Dickinson Vacutainer System, Plymouth, UK, 21-gauge needle). The preparation of PRP was performed within a maximum of 45 minutes after the blood collection and all aggregation studies were carried out within the following 3 hours. Citrated whole blood was centrifuged at 150 *g* for 10 minutes to prepare PRP, which was then stored at 37°C in a water bath. The platelet-poor plasma (PPP) was prepared by centrifugation of the pellet after the collection of PRP (1,500 *g* for 10 minutes).

Platelet aggregation

Platelet aggregation (PA) was performed in a four-channel aggregometer (PAP4, Biodata Corp., Horsham, PA, USA) purchased from MÖLAB (Hilden, Germany) and measured turbidimetrically by using different agonists according to the method described by Born (15). One hundred eighty-microliter aliquots of PRP were incubated at 37°C and platelets were stimulated by adding 20 μL of the agonist. Changes in light transmission (given as 0 to 100% aggregation) were recorded during constant stirring of the PRP at 1,000 rpm for 10 minutes. The values of the slope are given by electronic differentiation of the traces in the PAP4. It is based on the principles of a linear regression, using the largest decrease of the aggregation (%)/minutes (manufacturer's recommendations, [21]). Arachidonic acid, collagen, adrenaline (epinephrine), and adenosine diphosphate (ADP) were purchased from MÖLAB (final concentrations in the test tubes: AA, 500 μg/mL; collagen, 0.19 mg/mL; adrenaline, 100 μM; ADP, 20 μmol/L).

Drug administration

Blood was collected from patients before treatment with ASA in order to obtain pretreatment values. The ASA treatment in all patients started with a dose of 30 mg/day for 7 days (Miniasal®, Oranienburger Pharmawerk, Oranienburg, Germany). Aggregation was measured again, and if the AA-induced PA was more than 30% (inhibition of less than 70%), a higher dose of ASA (100 mg/day Aspirin Protect 100®, BayerVital GmbH, Leverkusen, Germany) was applied to the patients for a further week. PA was measured again, and those patients who did not show sufficient inhibition received 300 mg/day (Aspirin Protect 300®) for another week. In one case, the dose was increased to 500 mg/day for a week after control of PA.

Other laboratory parameters

During the hospitalization of the patients, the following laboratory parameters were performed routinely with the blood sampling for PA before ASA treatment: white blood cell, red blood cell, and platelet counts; hemoglobin; hematocrit; C-reactive protein; glycosylated hemoglobin; creatinine; total cholesterol; low-density lipoprotein; triglycerides; glucose; fibrinogen; and body mass index.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Correlations between parameters were analysed by unpaired and paired Student's *t*-test and $p = 0.05$ was considered as statistically significant.

The study was approved by the Ethics Committee of the Chamber of Physicians of the federal state of Thuringia/Germany.

RESULTS

Inhibition of PA by different doses of ASA

Platelets of all patients (N = 108) before ASA treatment responded to 500 μg/mL with an irreversible aggregation. The slope of the traces was 43.7 ± 1.4 (mean ± SEM) and the degree of the light transmission after 3, 5, and 10 minutes was 76.1 ± 1.9%, 83.2 ± 1.4%, and 86.3 ± 1.3%, respectively. One week after ASA treatment of all patients with 30 mg/day, PA in PRP was repeated. Forty-three patients showed a nearly complete inhibition of AA-induced PA, defined as an aggregation less than 30% at 10 minutes. All patients with a smaller degree of inhibition of AA-induced PA were treated with 100 mg/day of ASA for a further week. Arachidonic acid–induced PA was then inhibited with an aggregation less than 30% in 54 patients. In patients with less inhibition, the dose was increased to 300 mg/day of ASA for a further week. The inhibition of AA-induced PA was found to be sufficient in 10 patients after this dose. Only one patient had to be treated with 500 mg/day of ASA for a further week in order to inhibit AA-induced PA nearly completely. Figure 1 shows typical traces of AA-induced PA in a patient before ASA treatment and after the administration of 30 and 100 mg/day of ASA for 1 week.

The data of all patients were divided into three groups following the degree of inhibition of AA-induced PA as

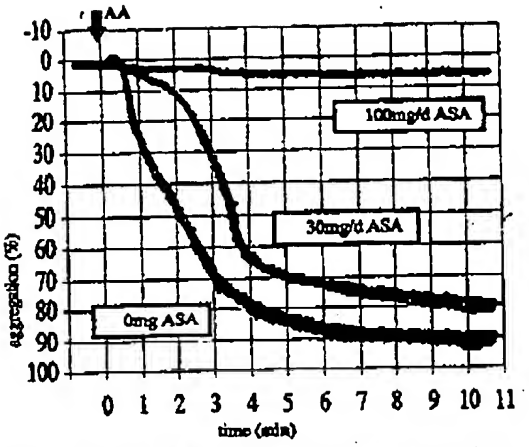

FIG. 1. Typical aggregation traces of a patient treated with different doses of acetylsalicylic acid (ASA). Arachidonic acid (AA)-induced platelet aggregation in platelet-rich plasma before treatment with ASA, 1 week after treatment with 30 mg/day of ASA, and a further week after treatment with 100 mg/day of ASA. Platelet aggregation is given as changes in light transmission. Final concentration of AA was 500 μg/mL.

shown in Figure 2: the "30-mg group" (n = 43), the "100-mg group" (n = 54), and the "300-mg group" (n = 10), respectively. The one patient who was treated with 500 mg/day of ASA was not included in the figure. Platelet aggregation in the 30-mg group before treatment with ASA was 76.2 ± 3.1%, 84.2 ± 2.3%, and 86.9 ± 2.2% at 3, 5, and 10 minutes after inducing aggregation, respectively. In all 43 patients in the 30-mg group, PA dropped down to less than 20% after treatment for 1 week with 30 mg/day of ASA. Fifty-four patients were included in the 100-mg group, and their PA was reduced from nearly identical pretreatment values compared with the 30-mg group to about 65 to 75% aggregation after the administration of 30 mg/day of ASA for 1 week, but to less than 20% after treatment with 100 mg/day of ASA for a further week. Ten patients were included in the last group, and their PA was reduced to less than 30% after treatment with 30 mg/day for 1 week, followed by 100 mg/day for another week, and finally with 300 mg/day for a further week.

In Figure 2b, the corresponding slopes of the aggregation traces are given. The slopes were reduced dramatically according to the inhibition of PA and a value of less than 10 can be used to describe nearly complete inhibition of PA.

Discharged patients continued taking ASA at home. Some of them (n = 23) were hospitalized again in the following 2 to 35 months (13 ± 2). Platelet aggregation was checked again in these patients (8 from the 30-mg group and 15 from the 100-mg group), and no differences in PA and in the slope of their aggregation traces were found to have occurred between the first and the second hospitalization.

Platelet aggregation in PRP was also induced with collagen, adrenaline, and ADP, and was investigated after treating the patients with ASA. The aggregation induced by these agonists was not completely inhibited as compared with AA as an inducer. The most sensitive parameter seemed to be the slope of the aggregation traces, which were summarized for the PA induced by different agonists before and after treatment of the patients with different doses of ASA in Figure 3.

Patients with diabetes mellitus

Among the 108 patients investigated, 50 had non-insulin-dependent diabetes mellitus (NIDDM). The behavior of their platelets was identical to that of patients without NIDDM. Nineteen diabetic patients belonged to the 30-mg group, 26 to the 100-mg group, and 4 to the 300-mg group; the one patient treated with 500 mg/day

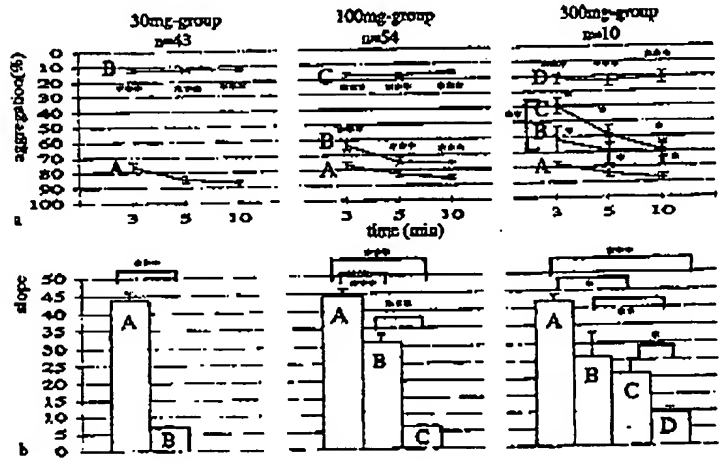

FIG. 2. Changes in light transmission (aggregation) (a) and slope (b) of arachidonic acid (AA)-induced platelet aggregation in patients treated with different doses of acetylsalicylic acid (ASA) (30, 100, and 300 mg/day, respectively). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control without ASA treatment in a). A = 0 mg; B = 30 mg; C = 100 mg; D = 300 mg.

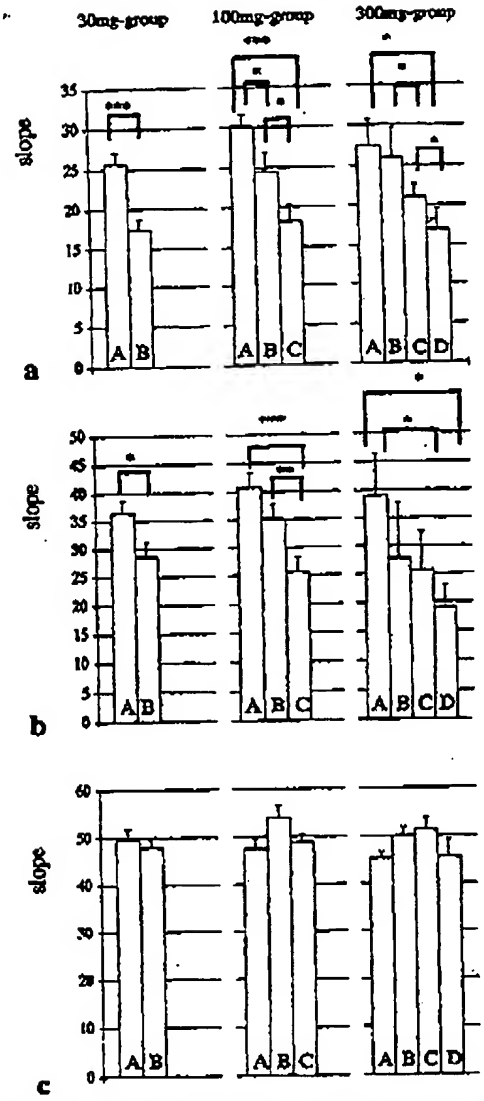

FIG. 3. Slope (± standard error of the mean) of the (a) adrenaline (100μM)-, (b) collagen (0.19 mg/mL)-, and (c) adenosine diphosphate (20μM)–induced platelet aggregation in patients treated with different doses of acetylsalicylic acid (30, 100, and 300 mg/day, respectively). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). A = 0 mg; B = 30 mg; C = 100 mg; D = 300 mg.

of ASA also had NIDDM. There was no difference in the percentage of nondiabetic and diabetic subjects in each group of patients. The data are summarized in Figure 4.

Platelet aggregation and other laboratory parameters

Platelet counts in whole blood did not differ significantly in patients over the time of treatment with ASA, and also did not differ between the three different groups (30-mg, 100-mg, and 300-mg). The statistical analysis did not show any clinically relevant predictive laboratory parameter concerning the inhibition of PA. Interestingly, increased levels in glycosylated hemoglobin were found in the three different groups of patients with NIDDM (7.44 ± 0.27%, 8.58 ± 0.4%, and 8.9 ± 1.6% in the 30-mg, 100-mg, and 300-mg groups, respectively). There was no correlation in the differences of the aggregation data concerning the use of other drugs (such as heparin, calcium-channel blockers, beta-blockers, angiotensin-converting enzyme inhibitors, or nitric oxide donators) that were given as comedication.

DISCUSSION

Acetylsalicylic acid is a widely used drug in the primary and secondary prevention of cardiovascular diseases. Studies have shown that low doses of ASA (30 to 100 mg/day) are sufficient to reduce the risk for cardiovascular events and do not differ significantly from higher doses (300 to 1,500 mg) applied (14,22–25). It is well established that the use of ASA as an antiplatelet drug is based only on the specific inhibition of cyclooxygenase activity, with a reduction or suppression of the thromboxane formation by platelets (5,7,26,27). The platelet aggregation described for the first time in 1962 by Born allows the specific ex vivo control of this platelet function (15). Arachidonic acid triggers the thromboxane formation in platelets including cyclooxygenase-1. The inhibition of this enzyme in platelets leads to an inhibition of AA-induced PA ex vivo. Therefore, it is

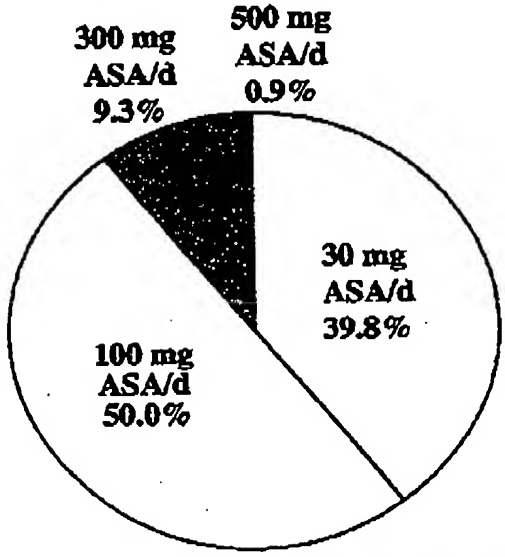

FIG. 4. Percentage of 108 patients with inhibition of the arachidonic acid–induced platelet aggregation by the administration of different doses of acetylsalicylic acid (ASA).

possible to control the inhibition of PA as a consequence of ASA in every patient using AA as an agonist.

Our study, involving 108 patients with a medical indication for ASA treatment, demonstrated that about 90% of the patients showed a nearly completely inhibited AA-induced PA when treated with 100 mg/day. This dose of ASA is widely used in Germany. It also shows that in 10% of the patients, PA was not sufficiently inhibited and that a higher dosage was required. Conversely, about 40% of the patients were overdosed with 100 mg/day of ASA. A control of the individual response to different doses of ASA is a simple, routine procedure that could lead to the treatment of patients with individualized doses of ASA. It remains to be established whether the nearly complete inhibition of AA-induced PA by ASA is correlated with risk reduction and the outcome of cardiovascular diseases. It also remains to be seen whether AA-induced PA or collagen-, adrenaline- or adenosine diphosphate (ADP)-induced aggregation is correlated with a clinically relevant platelet function inhibition. But PA may become a routine method in any hospital in daily clinical use. According to our results, an inhibition of AA-induced PA of more than 70% (aggregation less than 30%) and a slope of the aggregation traces of less than 10 are parameters indicating a sufficient effect of ASA.

We were unable to show any correlation of the laboratory parameters determined in this study with the degree of inhibition of AA-induced PA, indicating that none of the tested parameters has a clinically relevant predictive value concerning the inhibition of PA.

Other platelet agonists, such as collagen, ADP, or adrenaline, probably do not have any advantage compared with AA. There was a dose-response relationship as shown especially in the slope of the collagen- and adrenaline-induced PA concerning the dose of ASA. This shows the involvement of thromboxane formation in PA induced by different agonists, but in none of the different groups was there a stronger inhibition of PA than in the 30-mg group. The mean aggregation slope by inducing PA with different agonists could be used as sensitive marker for different studies (28).

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